Studies to reveal the patterns of activity in isolated spinal networks have recently evolved to include activity-dependent optical signals. We have used calcium sensitive dyes to optically track spinal network activity in real time. Associating electrophysiology with such imaging techniques has recently revealed spatiotemporal aspects of spinal networks responsible for rhythmic motor activity in the developing chick and mouse. In the present work several approaches have been applied for improving existing imaging procedures for labeling neurons with cell permeant and non-permeant calcium sensitive fluorescent dyes in spinal cords. Variations in optical emission intensity of labeled cells visualized through the ventral white matter or through cut face views into the grey matter were indicative of increases in dye-bound intracellular \([\text{Ca}^{2+}]\) that occur during cellular activity. Optical recordings were made of population activity and at the resolution of individual cells. Simultaneous ventral root recordings along the spinal cord were compared with the optical data to explain the spatial/temporal aspects of motor network output.

Although it has also been found that species-specific protocols must be developed, similar strategies were applied. The procedures reviewed for coaxing non-permeable calcium sensitive dyes into cells rely upon either retrograde transport from lesioned axon to soma or on introduction of transient cell membrane holes through electroporation, a technique typically used for gene transfection. Current research in this area focuses on optimizing labeling strategies to maximize quality (intensity, cell count, and labeled area) while maintaining spinal cord viability.

This review concludes that use of developing chick and mouse models for optical analysis of signal propagation and central pattern generation offer a very convincing approach for binding spinal cord anatomy to spinal network physiology. Optimization of current techniques and development of novel labeling strategies should prove fruitful in revealing cellular and network attributes responsible for spinally driven rhythmic spontaneous motor activity.
INTRODUCTION

If technologies such as neuroprosthetics or neural regeneration are ever expected to accurately reproduce normal motor behaviors, it is necessary to first understand the neural circuitry of the physiological motor systems being manipulated. We believe that developmental vertebrate spinal physiology has the potential to provide answers to the complex architecture and signaling of fast fully developed myelinated networks. Uncovering the endogenous activation of spinal networks should then allow for the most precise synthetic or modified activation of functional motor activity in walking vertebrates (Barbeau et al. 1999).

Besides offering the obvious advantages of understanding a naturally developing network when long term goals include redeveloping or bypassing certain neural connections that occur during development, developing spinal cords also permit an astonishing amount of experimental manipulation. We have chosen embryonic chick and neonatal mouse models for spinal network research for reasons detailed in Bonnot et al. 2001 loco. (the importance of transgenic model availability) and O’Donovan. 1989 (presence of reliable, well defined spontaneous locomotor-like bursting), but primarily because the central pattern generating networks responsible for locomotion in these species can be studied in isolated spinal cords in vitro. These small young spinal cords allow bath dissolved nutrients and oxygen to maintain even the most centrally located neurons. Isolated chick and mouse spinal cords exhibit robust rhythmic activity both spontaneously and upon evoked stimulation (Whelan et al. 2000). Whatever the approach may be, the goal of investigating spinal cord neuronal activity is to discover which neurons are involved in network activity, how they are involved, and the temporal distribution of these network events. Electrical activity in these preparations has been recorded in single cells via whole cell and patch clamp recordings, and in population recordings through lumbar ventral roots projecting off the spinal cord. Though one of these methods is extremely specific and the other is more generalized, even careful combinations of these electrical approaches fail to tell the whole story of the events that take place within the spinal cord during the activation of a complex network such as those responsible for generating locomotion. We have used calcium sensitive fluorescent dyes and ventral root electrical recordings as tools for monitoring entire groups of neurons simultaneously, while maintaining spatial resolution at the cellular level, of neuron location and morphology (McPherson et al. 1997).
Fluorescent labeling with Calcium sensitive dyes has already begun to elucidate signaling pathways during locomotive activity (O’Donovan et al. 1994; Bonnot et al. 2001 spat.). With this technique, information about a population of neurons as well as individual cells can be obtained. Hence, insights of network activity can be achieved where so far this has been a difficult task using traditional electrophysiological tactics. We have worked with Calcium Green dye varieties that increase in intensity of fluorescent light emission at a single wavelength as a direct function of calcium concentration (provided \([\text{Ca}^{2+}] < [\text{dye}]\)) (fig.2). Since some degree of intracellular calcium is always present (approx. \(1 \times 10^{-7} \text{M}\)) in spinal cells, Calcium Green works first as a histological label that allows visualization of all cells it has entered. From this sample of labeled cells, the selected cells involved in a network episode will increase in fluorescent intensity during an action potential when the cell depolarizes enough to trigger voltage gated calcium channels to open (AP intracellular \([\text{Ca}^{2+}]\): approx. \(1 \times 10^{-3} \text{M}\)). By digitally subtracting live high intensity digital video from an averaged image of a spinal cord labeled with calcium sensitive dye while electrically inactive, the percent change in fluorescence (\(\Delta F/F\)) could be calculated to determine activation of a region or single cell.

Optical recordings of cellular fluorescence fluctuations can be striking and unambiguous provided cells are well labeled. Here approaches for optimizing labeling are discussed in relation to specific aspects of motor activity that can be studied. Retrograde-transport labeling of neurons with dextran conjugates of Calcium Green involves the diffusion of dye through severed axons towards cell bodies. In the present work we have reviewed several applications of this technique as it applies to optical activity studies. The most significant limits to this technique are that it is restricted to neurons that have accessible axons that project to roots or run through the white matter, and any neurons that are labeled may be exhibiting somewhat abnormal behavior since their axons are lesioned and they cannot emit signals that may act as feedback for the network that activated them. Although a cell permeant form of Calcium Green does exist, it is difficult to work with, does not load well in bath applications, and must be dissolved in DMSO, which can be toxic at high enough concentrations (McPherson et al. 1997). We have however, included a successful approach for calcium imaging with the AM dye ester. Furthermore, previous work has shown that the dextran (Nance and Burns. 1990) and hexapotassium salt varieties of Calcium Green do not label well via bath loading or peripheral injections.
In this paper we show that dextran conjugated and hexapotassium varieties of Calcium Green can indeed be loaded into grey matter neurons without traveling through lesioned axons with the assistance of electroporation. Originally developed for the transfection of genes into specific tissues, and previously demonstrated for this purpose in neural tissue of embryonic chicks (Muramatsu et al. 1997; Nakamura et al. 2000), the practice of applying a high voltage gradient (5 to 25V) across living tissue in brief (50ms) pulses creates transient pores in lipid cell membranes that permit entry of charged molecules moving across the electrical gradient into the cytoplasm and nucleus. This method has previously been used to introduce the calcium sensitive dye fura-2 into fern spores for optical studies of calcium concentration changes (Scheuerlein et al. 1991). Put together, these studies warranted our efforts to examine electroporation as a spinal cord labeling approach for optical neurophysiology.

We have developed, modified, and reviewed a selection of approaches for achieving grey matter labeling of spinal neurons in the developing chick and mouse. We have not found a single method that may be deemed optimal, but rather have established a compilation of approaches that are tailored to species, spatial, and cell type specific parameters.

MATERIALS AND METHODS

Isolated Spinal Cord In Vitro Preparation

In order to have optimal access for electrical recordings and minimum interference for optical experiments, spinal cords were removed from neonatal (postnatal day 0-3; P0-P3) Swiss Webster Mice (Taconic Laboratory) from the tenth thoracic segment (T10) until the cauda equina using the technique described in Whelan et al. 2000. Dissections were done in perfusion chambers recirculating ACSF ([mM]: 128 NaCl, 4 KCl, 1.5 CaCl$_2$, 1 MgSO$_4$, 0.5 NaH$_2$PO$_4$, 21 NaHCO$_3$, 30 D-C$_6$H$_{12}$O$_6$) constantly bubbled with 95% O$_2$ and 5% CO$_2$. Most cords were removed at room temperature, and some were removed at 18ºC to preserve spinal function during longer dissection procedures.

White Leghorn chick embryos (embryonic day 8 to embryonic day 12) were decapitated and spinal cords were isolated from T3 according to the procedure in O’Donovan. 1989. Dissections were performed between 13 and 15ºC in recirculating Tyrode’s solution (Chick ACSF; [mM]: 139 NaCl, 2.9 KCl, 17 NaHCO$_3$, 12 D-C$_6$H$_{12}$O$_6$, 3 CaCl$_2$, 1 MgCl$_2$) constantly bubbled with 95% O$_2$ and 5% CO$_2$. Lumbosacral ventral roots were dissected along with the
cord in order to record the compound action potentials from motoneurons. If only optical experiments were being performed, all roots were removed during dissection.

**In Ovo Chick Electroporation**

In accordance with documented electroporation protocols for gene introduction, we adopted and modified an in ovo technique for labeling developing tissue and observing it after several days of additional development. White Leghorn chick embryos, embryonic day 2 (E2) to embryonic day 3 (E3), were exposed in ovo according to the procedure in Nakamura et al. 2000. In some chicks, the vitelline membrane was sliced open with fine scissors to enhance visualization of the spinal cord and allow easier access for injection. In E2 chicks neutral red dye was used to assist in visualizing the neural tube. Fast green (for enhanced contrast during injection) was added to calcium green conjugated dextran (10,000 MW; Molecular Probes; Eugene, OR) in 0.2% Triton (10mM), and this solution was pressure injected into the lumbosacral neural tube via a glass micropipette secured to a micromanipulator. Either 1mm or 3mm length gold gentrodes were positioned along the lateral surfaces of the embryo as described in Nakamura et al. 2000. Five electrical pulses lasting 50ms at 1s intervals were applied at various voltages across the gentrodes by an electro-square porator (BTX Inc; San Diego). Eggs were sealed with scotch tape and placed in an incubator until they reached E6 to E8 when their spinal cords were isolated and placed into a chamber perfused with Tyrode’s solution at room temperature for optical and electrophysiology studies.

**In Vitro Electroporation**

Bath immersion of isolated spinal cords in calcium sensitive fluorescent dyes allows for greater control over gentrode positioning and permits immediate recording before and after labeling. Mouse and chick isolated spinal cords were placed in a well with a volume of 100µL containing 5mM Calcium Green-1 hexapotassium salt (1147.19 MW; Molecular Probes) in room temperature ACSF. Either 1mm or 3mm length gold gentrodes (depending on size of spinal cord or area to be electroporated) were placed 3mm apart on either side of the parallel spinal cord. Most preparations were electroporated with the ventral face facing towards the anode to increase motoneuron labeling since motoneurons are found closer to the ventral
surface of the spinal cord. To maintain consistency and minimize the time our preparations were outside of ACSF perfusion, we designed a specialized chamber for these *in vitro* bath electroporation experiments (fig.2).

**Retrograde Dye Loading**

Another method of inserting cell membrane impermeable dye into cell soma takes advantage of an inherent property of lesioned axons to retrogradely transport certain molecules towards the cytoplasm. Using the principles established in O’Donovan et al. 1993, small incisions were made on one side of spinal cords in segment LS4 of isolated chick spinal cords in the ventrolateral funiculus (VLF) which consists primarily of ascending and descending axons. The cut portion of the VLF was drawn into a suction electrode that was back-filled with 6µL of calcium green dextran (10mM) in 0.2% Triton. The cords were left for 15–18 hours at 20ºC to label VLF projecting cell bodies.

Another form of retrograde labeling used to target muscle-innervating motoneurons was through muscular injection as described by Mentis et al. 1993. Dye is injected directly into the desired muscle (following general anesthesia of the animal) after an incision has been made in the skin. After Several days of retrograde labeling across the neuromuscular junctions and axons *in vivo*, it was found that motoneuron cell bodies were labeled. We injected calcium green dextran (10mM) into the Quadriceps, and Tibialis Anterior muscles of P0 mice and isolated the spinal cords at P2, P3, and P5.

**Grey Matter Microinjection**

To avoid grey matter neuron labeling through the white matter and protective spinal membranes, we created localized extracellular dye pools by direct injection into the grey matter. Spinal cords isolated from P2 or P3 mice were pinned down on a Sylgard *in vitro* chamber. Ventral or dorsal dura and pia matter were carefully removed from T11 to L2. Ventral root recordings were obtained to verify the presence of normal rhythmic activity. Using a sharp glass micropipette filled by with 3µL calcium green-1 AM (1,290.88 MW; cell permeant dye; Molecular Probes) in 20µL DMSO and 130µL ACSF (2.5mM) mounted on a
micromanipulator, 1 to 3 injections were made in the medial ventral or dorsal horn. Dye was slowly injected into the extracellular grey matter by air pressure regulated by a twist-style syringe.

**Aortic Cannulization**

Blood vessels throughout the spinal cord were filled with fluorescent dyes to take advantage of their proximity to neuronal cells. P2 mice were deeply anesthetized with methoxyflurane and then decapitated. Skin and limbs were removed before placing the preparation into the perfusion chamber (ACSF at 18°C). Pressure was applied to blood vessels near the point of decapitation by a thread tourniquet to minimize circulatory leakage of dye. The thoracic cavity was cut open and organs were gently pushed aside to reveal the descending aorta, which was then transected. Connective tissue around and under the exposed caudal face of the vessel was removed. An angled plastic suction electrode attached to a length of tubing was filled with ACSF and inserted into the descending aorta. A fine gauze string noose was used to clamp the vessel to the electrode. The vessel was slowly syringe flushed with 1mL of ACSF. Afterwards, the tubing and suction electrode were back-filled with either calcium green-1 AM (1,290.88 MW; 10mM) or calcium green-1 dextran (10,000 MW; 0.5mM) and dye was slowly pushed into the vessel (200µL). After detaching the cannula, the spinal cord was dissected free as described earlier.

**Optical Recordings**

Small changes in the intensity of fluorescent light emitted by calcium sensitive dyes as they bind calcium ions were recorded with a high intensity CCD camera (Stanford Photonics). Whole spinal cords or transverse segments cut with a fine razor were placed in a perfusion chamber with a glass coverslip bottom mounted to an inverted epifluorescence microscope (Nikon Eclipse TE300 or Nikon Diaphot). The spinal cords were perfused with ACSF at room temperature (25°C) or heated to 30°C to stimulate spontaneous activity. Excitation illumination was provided by a 75W Xenon light (excitation filter: 470-490nm, emission filter: 520-560nm), and CCD captured light was stored on SVHS media (Sony SVO 9500MD VCR). Changes in fluorescence were measured in real-time based on pixel intensity subtraction with frame
grabbing and image processing software (Metamorph; Universal Imaging; Downingtown, PA). These changes were averaged, normalized, and analyzed in Microsoft Excel. This protocol is documented in Bonnot et al. 2001 spat.

**Electrical Recording and Activation**

Simultaneous electrical signal recording accompanied optical recording. Tight fitting plastic suction electrodes into which ventral roots were drawn were used to detect changes over the DC bandwidth (DC-3kHz), and these data were digitized (Neuro-corder DR-886 or DR-890; NeuroData) and stored on tape (VHS). Suction electrodes could be switched from differential amplification of the electrical signals generated by compound action potentials from the population of axons bundled in a single ventral root, to stimulation of the tissue. Network activation was induced by constant current stimulation (Neurolog NL800 or World Precision Instruments A365 stimulator/isolator) of dorsal roots or the cauda equina (train: 4-20Hz, train duration: 5-10s stimulus duration: 500µs, strength: 20-100µA). Antidromic stimulation of motoneurons was performed by stimulation of suctioned ventral roots.

**Confocal Microscopy**

To confirm the cellular basis of optical activity and determine the location, amount, and type of cells labeled, we used confocal laser microscopy on thin sections of spinal tissue. Labeled spinal cords were fixed in cold EDC in PBS (50mg/ml; 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride; Sigma-Aldrich) for 4 - 24 hours. Subsequently, the spinal cord was embedded in 5% agar gel, sectioned horizontally (70µm) or transversely (50µm) with a Vibratome (St. Louis, MO), and mounted on slides covered by an antifading solution (PBS:glycerol 7:3) (Bonnot et al. 2001 spat.). Sections were imaged with the aid of a confocal microscope (excitation 488nm, emission 515-540nm ; Zeiss LSM410) within 24 after mounting. Images were line averaged (8 to 16), and z-series projections were generated in order to visualize the entire thickness of tissue (1-5µm intervals of up to 100µm of tissue).
RESULTS

**Retrograde motoneuron labeling – Introduction of Calcium Green dextran by muscular injection.**

Retrograde labeling experiments by injection into the Quadriceps, and Tibialis Anterior muscles of neonatal mice were confirmed by fluorescent microscopy. Ventral horn labeled cells with dendrites and soma diameters of 10-25µm were visualized. The location and spatial distribution of labeled cells was consistent independent of the time allowed after injection. Figure 3 shows the quality of labeling in three different spinal cords; each injected at P0 and allowed for retrograde transport to motoneuron somata at different points in time. The intensity and cellular resolution appeared to be a function of transport time. After five days, images of 70µm sections of spinal tissue show that labeling is specific to motoneurons. At high magnification labeling appears granular.

**Retrograde interneuron labeling – Ventrolateral funiculus fill**

Retrograde loading isolated chick spinal cords by surrounding a section of partially attached white matter with calcium green dye (fig.4A) targeted cells throughout the grey matter of the spinal cords rostrally to the location of the fill site whose descending afferents projected into the region being filled. These cells were expected to consist primarily of interneurons that serve to process sensory data and regulate motoneuron activity since motoneuron white matter is known to exist primarily in the ventral spinal roots and in spinal nerves. Additionally, electrical stimulation of the ventrolateral funiculus (VLF) in the lumbosacral spinal cord is known to initiate rhythmic motor responses indicating activation of a spinal motor network (Magnuson and Trinder. 1997). Three unique trials of VLF retrograde labeling with calcium green dextran were consistent in the number of neurons labeled (avg ipsilateral : avg contralateral = 705:229 stdev. 122:77) and the rostral spread of labeling (3-4 segments). Confocal microscopy images (fig. 4B) of horizontal sections were used to determine, based on size, shape, and location that few or no motoneurons were labeled, and that the neurons labeled were similar in size and shape suggesting homogeneity of the type of cells labeled. Labeling
was primarily concentrated on the half of the spinal cord ipsilateral to the fill site, but labeling was also found on the side contralateral to the fill.

*Grey matter microinjection*

To examine the potential for localized placement of calcium sensitive dye into a segment or specific region of the mouse spinal cord, the cell permeable AM form of Calcium Green was used for injections of pools of dye into the extracellular grey matter for diffusion into neuronal cell bodies. Figure 5A shows the relative distribution of labeling for three of these localized injections. Optical calcium signals were monitored during stimulation trains administered to the cauda equina to show that dye had successfully entered active neurons involved in rhythmic motor activity. Changes in fluorescence (fig. 5B) were recorded for each injection region, and spontaneous as well as stimulus evoked events were recorded from ventral roots such that the relationship between electrical activity and fluorescent intensity could be seen (fig. 5C).

*In Ovo electroporation*

To test the feasibility of electroporation of spinal neurons with calcium sensitive dyes as a technique for fast, specific labeling while maintaining electrical signal viability we performed *in ovo* electroporation of the embryonic chick spinal cord after injecting calcium green-1 dextran into the central neural tube. The dorsal surface of the chicks were electroporated with 5, 50ms pulses of 25V current at 1s intervals. After 4 to 5 days of post-electroporation incubation, the survival rate of these chicks was low (1 out of 13), but since no control chicks survived, we were not able to determine the percent of fatalities related to electroporation versus other aspects of the procedure. Isolated spinal cords from surviving chicks demonstrated normal spontaneous and evoked electrical activity, and were optically active as well (fig. 6C-D). Fluorescence microscopy of an *in ovo* electroporated spinal cord revealed residual neural tube labeling, as well as a clear unilateral bias for the presence of the negatively charged calcium-1 green dextran dye in the side of the spinal cord nearest the positively charged gentrode (fig. 6A). Cellular labeling ranged from the dorsal white matter to the medial
grey matter (fig. 6B), and although no motor neurons were labeled, this was most likely due to their ventral horn location lacking proximity to the dorsal site of electroporation.

**In Vitro electroporation**

Electroporation of the isolated chick spinal cord bathed in Calcium Green-1 hexapotassium salt proved to be a fast procedure with options for manipulation (gentrode placement, dye concentration, and electroporation voltage and pulse number), and a low fatality rate. Cords electroporated in vitro often required 1 to 2 hours for surface dye to wash out and for motor network activity to fully recover from the procedure. Figure 7 highlights the effects of electroporation and bath concentration on segments from a chick spinal cord. Widespread labeling and preservation of rhythmic electrical activity upon dorsal root stimulation made this technique a good candidate for analysis of spatiotemporal activation of the developing spinal network responsible for spontaneous developmental movement (Ho and O’Donovan. 1993). The use of discrete regions of interest in a rostral-caudal array for optical analysis of the spatiotemporal propagation of network component activation is shown in figure 8.

Although similar approaches to *in vitro* electroporation in the mouse spinal cord have yielded good neuronal labeling as seen in confocal microscopy images, we concluded that although labeled, these cells were no longer playing an active role in network activity (which also experienced a net decline as shown by electrical activity deficits) since no optical signal was produced from these cells during spontaneous or stimulated episodes. Preliminary investigations suggest that successful entry of Calcium Green-1 hexapotassium salt into neurons by electroporation has a toxic effect on those cells. When voltages necessary to achieve labeling (10-20V) were used in baths of Calcium Green, very few spinal cords were able to resume normal evoked electrical rhythmicity even after several hours of recovery. But if high voltages (up to 30-40V) were applied without dye, or if dye (5mM) was applied without current, activity returned to normal rhythmic baseline shortly after manipulation.

**Aortic cannulization**

By cannulizing the descending aorta over the lower thoracic spinal column, flushing it with saline, and pushing Calcium Green-1 dextran through, we were able to load many of the...
spinal arteries in the neonatal mouse with dye (fig. 9A). After isolating the vessel-filled spinal cord, we electroporated (8 pulses, 15V, 50ms duration, 1s interval) the cord to coax the dye out of small blood vessels and into nearby neurons. Confocal microscopy images of horizontal sections from a cannulized/electroporated spinal cord indicated that a large area of neurons was labeled and that both motoneurons and interneurons were successfully labeled (fig. 9C). Since the only extracellular space labeled significantly was spinal blood vessels, individual cells could be visualized with good contrast through the white matter of the cord while in the optical/electrical recording chamber (fig. 9B).

**DISCUSSION**

Understanding how cells in the spinal cord coordinate in order to produce patterned motor activity lies in uncovering spatiotemporal propagation of that activity. Through the combination of electrical recordings from groups of motoneuron axons in the ventral roots and real-time calcium imaging of network recruitment during spontaneous and electrically evoked episodes, we have shown that spinal cord physiology may best be understood by visualizing which neurons are responsible for the electrical waveforms recorded from various sites along spinal cords. A variety of approaches have been presented for introducing calcium sensitive dyes into spinal neurons, and each approach has been designed to answer different questions about network activity.

Injecting calcium sensitive dye into the muscles of neonatal mice has been shown to provide specific motoneuron labeling for the cells that directly innervate the muscles injected. This technique allows for the establishment of the location of cells directly responsible for flexor/extensor activity, and also allows for optical *in vitro* studies that can explain which muscles receive commands at any time during an electrical episode. Although cellular labeling is present two to three days after such an injection, the procedure currently produces optimal cellular contrast somewhere near five days after injection. However, because deep grey matter anoxia occurs with P5 spinal cords *in vitro*, they are not used for real-time analysis, but are excellent for histological mapping. To visualize the activity of retrogradely labeled motoneurons in relation to the interneuron networks responsible for their activation, other labeling procedures may be applied to these spinal cords as well.
We successfully applied the retrograde fill strategy to quantifiable, consistent labeling of interneurons projecting axons through the ventrolateral funiculus. This was a good method for illustrating spatial distribution of cells both contralaterally and ipsilaterally located to the fill site, projecting to a selected surface section of white matter. Contralateral labeling, while 75% less than ipsilateral labeling, is still remarkable since only cells ipsilateral to their VLF projections have previously been shown to be responsible for locomotive network activation (Yamaguchi. 1985). This procedure does risk some degree of network degradation from long fill times and severed axons of cells being studied. Nevertheless, the VLF retrograde fill approach allows real-time optical studies with high cellular contrast, low background (except for heavy axon fluorescence near the fill site), and a moderate rostral-caudal spread (3-4 lumbosacral segments).

Experiments were primarily performed using dyes incapable of traversing cell membranes, although an ester form of Calcium Green that is capable of diffusing through neuronal membranes does exist. Despite it’s acclaimed permeability, previous attempts to load the Calcium Green AM dye in lamprey spinal cords have not been fruitful (McPherson et al. 1997). Localized injections of cell permeable Calcium Green AM were attempted after bath loading electroporation experiments with cell impermeable dyes failed to produce live optical signals in the mouse. Injection loading was advantageous in that for the most part it reduced strong white matter labeling often seen in bath loaded preparations, thus reducing the background fluorescence of network cell bodies being viewed in the grey matter through the white matter. Activity recorded from ventral roots along the spinal cord seemed robust even after three separate injections or recovered shortly after an injection. Injecting with fine tip glass micropipettes allowed very precise placement, but constraints on injection volume were imposed by the tendency of the AM dye to precipitate and clog the electrode tip. Though sufficient for regional location of active cell populations, the percent change in fluorescence in spinal cords injected with the cell permeable ester was quite low. Further development of this procedure must focus on more consistent penetration depths into the grey matter as well as preventing tip blockage by dye crystals. An inevitable drawback to this approach is that since dye is deposited via injection, the area being studied will have experienced some degree of presumably minimal damage.

The implementation of a strong electrical gradient across spinal tissue to force large charged dye molecules into the intracellular domain was successful enough in our chick
experiments to warrant further investigation of this seemingly flexible labeling concept. Electroporation has the potential to provide additional loading attributes to the current stock of labeling techniques; primarily the ability to load intrinsic cells that may not have axons that project out of roots or span the funiculus (O’Donovan et al. 1993) and 3-dimensional control over the location of loaded cell populations by gentrode positioning. Electroporation can also overcome the time course required for retrograde transmission of dye into cell somata. In muscular fills this waiting period can prevent examination of a sufficiently labeled cord at the desired age, and in VLF, nerve, or ventral root fills in isolated spinal cords, the spinal tissue can began to degrade during the time required for adequate labeling. If applied to animals at later stages, electroporation might also prove to overcome the 1-2cm diffusion limit of retrograde labeling (O’Donovan et al. 1993). We have confirmed that electroporation allows for fast cellular loading of neuronal cells (interneurons, and motoneurons) as well as non-neuronal cells (glial), while preserving network function as determined by optical and electrical recordings of neuronal activity.

Electroporating the chick spinal cord in ovo illustrated the polar loading nature of the technique, and allowed for isolation of spinal cords without the need for recovery or surface dye washing periods. The in ovo approach produced good fluorescent signals, and exhibited minimal background fluorescence other than in the neural tube. Two remarkable drawbacks to the in ovo approach as used here remain. The spinal column of the in ovo chick is only accessible for gentrode position on areas lateral to the dorsal spinal cord. Since the lateral motor columns run through the ventral horns, this positioning fails to target these areas. Secondly, the very low survival rate of injected embryos (not necessarily electroporated) was discouraging.

In vitro bath loading of Calcium Green dye using electroporation allowed for thorough labeling of interneuron and motoneuron populations. Enhanced labeling in desired regions of the spinal cord could be achieved by placing the anode near the target area. This worked presumably because dye would move slightly during each pulse away from the anode, and into the spinal neurons as it headed towards the cathode. Dye concentration was found to affect the quality of labeling for in vitro electroporation, with optimal loading occurring between 5-10mM. Though similar labeling with this approach took place in the isolated mouse spinal cord, the inability to record normal motoneuron output from the ventral roots, and the lack of any significant change in fluorescence intensity in most preparations prompts further
investigation. Controls omitting either electroporation or Calcium Green-1 hexapotassium salt exclusively suggest that successful cellular labeling via electroporation of Calcium Green-1 hexapotassium salt and maintained network activity are paradoxical. The failure of this approach in mice preparations is puzzling, especially since there is no evidence to suggest that the dye has a chemically toxic mechanism once inside a cell. It is not yet known whether electroporation with other forms of Calcium sensitive dyes will involve similar network dysfunction in in vitro mouse experiments.

The difficulties encountered with in vitro electroporation in neonatal mice impelled testing alternative approaches for utilizing electroporation in mice while preserving network activity. We hoped to minimize the application of the factors that additively had an adverse effect on network activity (high voltage current and Calcium Green dye). The principles behind filling spinal blood vessels with dye were that lower concentrations of dye could be used along with lower electroporation voltages and pulse numbers since the dye would be located in small vessels very proximal to the cells that we desired to be loaded. It may be possible for electroporation and calcium green to be used together in mouse spinal cords, provided this dichotomy is in proper balance. If the procedure can be optimized so that the spinal cord is not deprived of oxygen for too long while in the vertebral column and activity losses similar to the in vitro experiments are not encountered, this approach should provide good cellular resolution with low background fluorescence in the regions directly surrounding cells. However, net background fluorescence will still remain high due to emittance from filled vessels.

Monitoring neuronal calcium concentration changes optically while recording electrical output from motoneuron populations offers an opportunity that electrophysiology alone has never been able to offer. With the techniques detailed here, the population activity of interneurons may be revealed in direct relation to motoneuron population activity. We have shown that it is possible for single action potentials to be detected in individual cells, and the spatial propagation of action potentials underlying the activity of central pattern generators can be observed across time. In comparison to alternative labeling approaches for visualizing spinal activity with voltage sensitive dyes, calcium sensitive dye approaches allow for stronger optical signals and cellular resolution. Therefore, we feel efforts to overcome the numerous challenges presented regarding the loading of these dyes are justified. Future studies should be structured to enhance labeling on two fronts. First, improving techniques to achieve maximal
labeling of all neurons in a desired area with minimal background fluorescence. When we are confident that most cells in an area are labeled, it decreases the number of neurons that could become active without producing an optical signal. Conversely, achieving very specific labeling when desired could enhance our confidence about network function by allowing us to ask whether or not neurons at certain locations in the 3-dimensional space of the spinal cord are active during locomotive-like motoneuron output. In any approach, minimizing background fluorescence, maintaining normal electrical network output, and enhancing loaded cell contrast are key. Additional focus on improving the methods detailed here as well as the development of novel loading approaches will unravel many of the mechanisms involved in generating locomotion-like patterns.

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**References**


**Figure 1**
**Calcium Green Mechanism:** The BAPTA binding moiety serves to block the intrinsic fluorescence of Di-Chloro Fluorescein, and contains the calcium binding site. Upon binding with calcium, the aminodiacetate group of BAPTA is less likely to transfer a fluorescence-quenching electron to the fluorophore, allowing the fluorophore to emit a photon. This mechanism allows for a constant wavelength emission at various intensities limited by calcium concentration. The molecule shown (Ca²⁺ green-1 hexapotassium salt) has a molecular weight of 1147.19, and is cell impermeable under normal conditions. The peak absorption wavelength for this molecule is 506nm and maximum emission occurs at 531nm (Yuste et al. 2000; Molecular Probes. 2001).

**Figure 2**
**In Vitro Electroporation Chamber:** A diagram of the chamber designed for isolated spinal cord electroporation. Closing both shunts created a 100µL chamber that dye could be added to, and the cord could be electroporated in. Immediately after electroporation, shunts were opened and normal oxygenated ACSF flow resumed to nurture and wash surface dye off the spinal cord. The bottom of the chamber consisted of a glass coverslip that allowed optical recordings with an underviewing microscope.

**Figure 3**
**Retrograde Muscular Injection:** Calcium green injections into various hindlimb muscles in the mouse were used to target specific motor neuron pools that innervated the muscle groups injected. The figure contains 70µm horizontal sections (20x) from three mice, each injected in the quadriceps at postnatal day 0 (P0) with calcium green-1 dextran. A: This cord was isolated from a mouse 2 days after injection. Motoneurons can be seen but labeling is faint. B: The mouse dissected at P3 shows higher intensity labeling. C: Five days after injection the motoneuron labeling is distinct and bright.
Figure 4

VLF Retrograde Fill: The property of lesioned axons to convey dye to their somas was taken advantage of to transport calcium green-1 dextran dye to interneurons that project axons through the ventrolateral funiculus (VLF) in the lumbar spinal cord. A: Diagram of the fill procedure. Spinal cords are left to fill like this for 15-18 hours while in chilled oxygenated ACSF. B: Confocal image of 70µm horizontal section from an embryonic chick (consists of two merged projections at 10x; each z-stack projection represents the entire section taken at 4µm intervals). Cellular labeling is present mostly on the side ipsilateral to the lumbosacral segment 5 fill site (LS5), but contralateral labeling is also present, especially distal to the fill site.
Figure 5

Grey Matter Injection with Cell Permeant AM variety of Calcium Green: Three microinjections of calcium green AM dye were made in the ventromedial grey matter of this neonatal mouse spinal cord. A: The three white areas seen on this image taken while the cord was in the recording chamber represent the three injection sites. Regions of interest were defined by subtracting this frame from an image of the spinal cord during stimulation. Although purple region number 4 has very low resting fluorescence, subtraction showed that it displayed a significant change in optical intensity during network activation. B: Histogram of fluorescent intensity fluctuation for each region. Time is on the scale of 30 frames/sec. The optical signal is coupled to C: The electrical responses recorded from a lumbar ventral root during an episode of spontaneous origin.
Neural Tube Injection and Electroporation: Microscope images showing stimulus responsive changes in fluorescence. This chick spinal cord was labeled with a calcium green injection into the neural tube and in ovo electroporation (25V, 5 pulses, 50ms duration, 1s interval) at E3. The spinal cord was isolated for analysis at E7. A: Ventral surface of the lumbar cord. Neural tube is visible as vertical white line in the center. Anode and cathode locations are shown in relation to dye distribution (calcium green-1 dextran carries a negative net charge). B: 50µm transverse section taken from region indicated in A. Unilateral distribution of labeling apparent in the 10x section. At 20x, the spatial-specific labeling of neurons is clear. C: A pseudo-colored fluorescence intensity difference image produced by subtracting A from a frame grabbed while the motor network was activated. D: Normalized and averaged histogram of the fluctuations of fluorescent intensity in two regions of the lumbar cord. Time scale = 30frames/sec.
Figure 7

Labeling as a Function of Concentration in Chick Spinal Cord In Vitro Electroporation: Confocal microscopy reveals labeling in chick spinal cord segments electroporated in baths of Calcium green-1 hexapotassium salt at 3mM and 10mM concentrations. Transverse segments from the lumbar region of an isolated chick spinal cord were electroporated at 25V. A: A control segment was placed in 10mM of dye for 5 min (amount of time all segments were in dye) and then washed in ACSF. Some faint motoneuron labeling is present in a ventral horn B: Segment was bathed in 3mM dye during electroporation. Some superficial cells in the white matter are clearly labeled, and several motoneurons are very faintly labeled C: Segment was bathed in 10mM dye during electroporation. There is bright labeling of some cells in the white matter as well as good motoneuron labeling in the ventral horn.
**In Vitro Bath electroporation**: This isolated E10 chick spinal preparation was placed in a bath of 5mM Calcium green hexapotassium salt and electroporated with 20V. 

A: Regions of interest overlaid on the image of the ventral lumbar spinal cord during optical recording. 

B: Pseudo colored difference image created by the subtraction of A from a frame grabbed during a rhythmic motor network episode. 

C: Normalized and averaged histogram of the optical activity recorded during a stimulus induced rhythmic bursting episode. Each trace represents the change in fluorescence intensity for a specified region of interest. 

D: Normalized close-up of highlighted region in C. The order of activity onset suggests a rostral-caudal propagation of motor network activation.
Aortic Cannulization and Electroporation: A cannula inserted into the descending aorta of a neonatal mouse was used to introduce the cell impermeable dye, Calcium green-1 dextran. The isolated spinal cord was then electroporated (15V) to promote cellular uptake. A: Dye filled dorsal blood vessel in the thoracic spinal cord. B: View of individual cells through the white matter of the cord in the recording chamber. C: 10x confocal view of 70µm horizontal section. Labeling is widespread and individual neurons can be easily resolved (overlay is 25x z-stack projection of the entire section at 5µm intervals).